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The project reported here focuses on the regulation of neuronal sensitivity by a novel class of protein kinase: an ecto-protein kinase which phosphorylates proteins at the cell surface by extracellular ATP. We proposed that the ecto-protein kinase that our previous studies have described in neuronal cells may play a significant role in the regulation of neuritogenesis and synaptogenesis. Our studies are designed to provide experimental evidence in support of this hypothesis. The progress we have made in this project includes the development of novel experimental paradigms for the determination of ecto-protein kinase and its substrates in cultured cells. We used these paradigms in the conclusive identification of the surface phosphoproteins in primary neurons cultured from embryonic brain and in PC12 cloned neuronal cells induced to differentiate by nerve growth factor (NGF). We have determined which of these surface phosphoproteins are regulated by NGF. We have also identified a specific phosphorylation system at the surface of CNS neurons involved in neurogenesis. These specific phosphoproteins should be at the focus of continued studies on the role of ecto kinase in synaptic plasticity.

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PROGRESS REPORT AND FORECAST

Period of this report: April 1, 1990 to December 31, 1991

Principal Investigator: Yigal H. Ehrlich, Ph.D.

Concise Summary

The goal of our research is to determine the role of surface protein phosphorylation by ecto-protein kinase activity in neuronal development and synaptic plasticity. In this research we use two model systems. The first is a homogeneous population of cloned cells of the line PC12 and the induction of neuronal differentiation in these cells by nerve growth factor (NGF). A major aim of this project has been to complete the detailed characterization of surface protein phosphorylation in PC12 cells and to determine the mode of regulation of this activity by NGF. This goal has been accomplished. A manuscript entitled: "Ecto-Protein Kinase and Surface Protein Phosphorylation in PC12 Cells: Interactions With Nerve Growth Factor" (10 figures and 3 tables) has been prepared and accepted for publication (pending some revisions) in the Journal of Neurochemistry. The manuscript acknowledges the support by AFOSR grant #88-0290, and a copy is enclosed with this report.

In parallel to the study of PC12 cells, we have continued our investigation of the developmentally regulated surface phosphorylation of proteins in primary cultures of neurons from embryonic chick telencephalon. We completed to collect the evidence that these neurons possess ecto-protein kinase and to identify its specific substrates. We have carried out detailed characterization of this activity, identified specific surface phosphoproteins implicated in neuritogenesis and initiated experiments designed to purify these surface phosphoproteins.

Research Objectives

The specific aims and objectives of the research carried out in this project can be summerized as follows:

(1) Biochemical characterization of the extracellular protein phosphorylation systems operating in PC12 cells and in primary CNS neurons differentiated in culture. This objective includes experiments demonstrating conclusively the ecto-enzymatic nature of the protein-kinases under investigation.

(2) Identification and characterization of the surface neuronal proteins whose phosphorylation by extracellular ATP alters during neuronal development and synaptogenesis.

(3) Isolation and purification of a neuronal ecto-protein kinase and of specific surface proteins that serve as its substrates, and the preparation of antibodies against these components. Emphasis to be placed on obtaining inhibitory antibodies.

(4) Use the tools and probes prepared above in investigation of the involvement of ecto-protein kinase in the regulation of neuronal function by extracellular ATP, with emphasis on studies of neuritogenesis and synaptic plasticity.

Research Accomplishments During the Report Period

Our progress in studies on the interactions of NGF with the ecto-protein kinase systems in PC12 cells is detailed in the manuscript enclosed with this report. Our progress in studies of the ecto-protein kinase systems in developing CNS neurons is summerized below.

The previous Progress Report of this project has demonstrated that surface proteins migrating with apparent MW of 105K, 13K and 11.7K are exclusive substrates of ecto-protein kinase in primary chick brain neurons. The experiments designed to characterize this activity during the present report period have focused on the developmental regulation of this enzymatic system.

To further characterize the extracellular nature of the neuronal ecto protein kinase, the exogenous substrate casein was utilized. We have found that casein is phosphorylated when added to a 10 min phosphorylation reaction carried-out with intact cells, further indicating that the ecto-protein kinase is indeed extracellular. A significant difference was seen in this extracellular phosphorylation activity

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between immature neurons grown in-culture for 2 days, and mature neurons grown in-culture for 6 days. Immature neurons were observed to have greater ecto-protein kinase activity than differentiated 6 day neurons. The phosphorylation of the endogenous substrates 11.7K and 13K proteins, as well as phosphorylation of the exogenous substrate casein, by extracellular [γ-^{32}P]ATP is much higher with 2 day neurons than in 6 day neurons, suggesting greater ecto-kinase activity in the earlier stages of development.

The time course of the phosphorylation of the 11.7K and 13K protein substrates by ecto-protein kinase in immature 2 day cells was compared to mature 6 day cultured neurons. Two day primary neurons displayed greater ecto-protein kinase activity than did 6 day neurons over all time points studied for both the 11.7K and 13K proteins. The incorporation of phosphate increased in 6 day neurons for both the 11.7K and 13K proteins for up to about 20 min and then reached plateau. Initial incorporation of label into the 13K and 11.7K proteins of 2 day neurons increased at a faster rate and reached plateau for the 13K protein, but labeling of the 11.7K protein continued to increase for up to 60 min. These extracellular protein phosphorylation systems are thus developmentally regulated, with all the measured kinetic parameters of enzymatic activity showing enhancement in the earlier phase of development.

To assess the specificity in the activity of ecto-protein kinase in primary CNS neurons, we examined the effects of inorganic phosphate. The addition of unlabeled Pi should specifically dilute the labeling by ^{32}Pi, but should have no effect on labeling of extracellular proteins by [γ-^{32}P]ATP. We found that the addition of 1 mM unlabeled Pi to reactions carried-out with primary neurons eliminated all labeling by ^{32}Pi for both the 10 min and the 60 min reactions. Protein bands labeled specifically by [γ-^{32}P]ATP, especially the 11.7K and 13K proteins, were <u>unaffected</u> when unlabeled Pi was added to either the 10 min or 60 min reactions. It is noteworthy that phosphorylation of the 11.7K and 13K proteins is greater in 2 day neurons than in 6 day neurons, especially during the initial time points of the reaction, both in the initial rate and extent of phosphorylation. To investigate further these developmental changes, the phosphorylation pattern of proteins was determined in 2, 3, 4, 5, 6 and 7 DIV neurons incubated with extracellular [γ-^{32}P]ATP or ^{32}Pi for 10 min. The labeling of the 13K and 11.7K protein bands increased up to day 3

followed by a distinct reduction in the labeling of these two proteins by extracellular ATP. A quantitative analysis of these data has provided the unique developmental pattern of the extracellular phosphorylation of the 11.7K and 13K proteins. Accordingly, we have decided to focus the continued research on these two specific surface phosphoproteins.

The effect of divalent cations on endogenous ecto-protein kinase activity were determined. Primary neurons were incubated with a buffer containing Ca^{++} and Mg^{++}, Ca^{++} only or Mg^{++} only, and labeling of neurons with [γ-^{32}P]ATP for 10 min was assessed under these conditions. When Mg^{++} is removed (Ca^{++} only buffer), the protein bands of MW 11.7K, 13K, 64K and 105K are not labeled. Phosphorylation of the 17K band increases in Ca^{++} only buffer conditions. This data indicates that the ecto-protein kinase responsible for the phosphorylation of the 11.7K and 13K proteins is a Mg^{++} dependent enzyme. Using the methods and procedures described in the enclosed manuscript, we have determined that over 90% of the phosphate transferred by ecto-protein kinase activity from extracellular [γ-^{32}P]ATP to the 13K and 11.7K proteins is incorporated into phosphothreonine. Thus, as in PC12 cells, the ecto-protein kinase in primary CNS neurons in also a Mg^{++}-dependent threonine kinase.

The effects of the protein kinase inhibitors K252a and K252b on protein phosphorylation in primary neurons were examined this year. A significant reduction in the phosphorylation of all proteins occurred in neurons pretreated with either K252a or K252b for 10 min and then incubated with [γ-^{32}P]-ATP for an additional 10 min. Both K252a and K252b are protein kinase inhibitors. However, while K252a can cross the plasma membrane, it has been reported that K252b can not penetrate intact cells. Thus, only K252a, but not K252b, should inhibit intracellular protein phosphorylation. Indeed, when primary neurons were treated with K252a, we found that there was a very significant reduction in the intracellular labeling of all proteins. In contrast, K252b had virtually no effect on the phosphorylation of intracellular proteins. Our data therefore confirms that K252b will not cross the plasma membrane and when applied to intact cells, it is a <u>selective</u> inhibitor of ecto-protein kinase activity. This inhibitor can now be used directly in developmental studies designed to determine the function of ecto-protein kinase in neuritogenesis.

To determine the effect of various gangliosides on ecto-protein kinase activity, primary neurons were pretreated with 5nM of gangliosides for 1 hour and then incubated with [γ-^{32}P]ATP for 10 min. The phosphorylation of the 11.7K and 13K proteins, the specific substrates of ecto-protein kinase, was significantly stimulated by GM3. The monosialo-gangliosides GM1 and GM2 had similar stimulatory effects on phosphorylation of these two proteins. GD1a, a disialoganglioside also had stimulatory effects on the phosphorylation of the 11.7K and 13K proteins. The gangliosides tested in these experiments were shown in previous studies to stimulate neurite extension. Their selective stimulatory effects on the extracellular phosphorylation of the specific protein substrates of ecto-protein kinase further support our suggestion of a role for this enzymatic system in neuritogenesis, and indicates that detailed investigation of this system can reveal novel and important regulatory mechanisms operating during neuronal development.

In experiments carried-out this year we have determined that endogenous ecto-protein kinase activity in dissociated cells prepared from the telencephalon plus olfactory bulbs of 7 day chick embryos, was qualitatively the same as that seen in attached cells assayed after 2 days in culture. These assays were carried-out with cells in suspension using the standard reaction buffer. Thus, for the PURIFICATION of surface phosphoproteins we are not limited by the need to culture telencephalon neurons and by the limited amount of cells that can be processed with this procedure. Instead, we shall use as starting material for ecto-protein kinase isolation, and for isolation of endogenous substrates, dissociated cells from whole forebrain. The number of eggs to be used is NOT a limiting factor. In this context it is also important to determine that the dissociated ecto-protein kinase can phosphorylate the 11.7K and 13K surface proteins which are at the focus of this project. As described below, we now have a procedure for preparing a soluble fraction containing these two proteins. Thus, before carrying out large scale purification, sequencing and cloning of the ecto-kinase, we shall use these isolated proteins as exogenous substrates in in-vitro assays of the isolated enzyme. Furthermore, due to the progress in our efforts to isolate the 11.7K and 13K surface phosphoproteins, we plan to concentrate our efforts on substrate purification in the next stage of this research. In subsequent work designed to isolate the ecto-protein kinase, purified 11.7K and 13K protein will be used as exogenously added substrates in key test assays during kinase purification.

From the experiments in which surface-phosphorylated 11.7 and 13K proteins of chick embryo primary neurons were electroeluted to determine their phosphoamino-acid composition, and using the specific activity of the added [γ-^{32}P]ATP and the number of reacted cells, we have estimated the yield of electroeluted protein in the final step. A typical reaction in this procedure was carried-out in a 6 well-cluster plate with $5x10^6$ cells per well. Reaction products from 5 wells were electrophoresed in 4 gels and final products of each band were concentrated in 400µl. From aliquot countings, using the specific activity of the ATP and assuming that the surface protein is phosphorylated at a single site, we have calculated that the final preparation of 11.7K-electroeluted-material (400µl) contained an estimated total of 0.28 pmole protein derived from 25 x 10^6 cells in the reactions providing this product. Based on cell counts, this would translate to a yield of 0.34 pmole per telencephalon, and to 1.36 pmole per whole brain. Thus, to obtain 50 pmoles in the final preparation, only 36 embryos are needed as starting material. This is a most feasible undertaking as in our routine operation we process four dozen (48) eggs per dissection. Although feasibility is readily demonstrated by this estimate it should be emphasized that this calculated yield represents, in all likelihood, a large UNDER-estimate. The CPMs measured in the electroeluted material used for phosphoamino-acid determination were derived from a 10 min reaction; a 60 min reaction period will provide a better count of the total number of molecules, and that would be 2.5 times greater than our conservative estimate. Furthermore, as indicated above we have now determined that the pattern of the 11.7K and 13K protein phosphorylation by extracellular [γ-^{32}P]ATP added to dissociated cells of 7-8 day chick embryo brain is the same as that with immature cultured neurons. Therefore, the reactions used to tag the 11.7 and 13K proteins by ^{32}P at the neuronal cell surface will be carried-out in suspension without the need for cell plating. The decreased effort and time involved will enable more frequent processing and greater yield of purified proteins.

Development of alternate approach for purification of the 11.7K and 13K protein substrates of ecto-protein kinase: We have obtained data that provide a new procedure for purification of the surface phosphoproteins at the focus of this project. In brief, neurons from the telencephalon of 7-days chick embryos were plated in 48 well cluster plates at 22 x 10^4 cells/well and the medium changed to chemically-defined ITS+ 20 hrs

after plating. Ecto-protein kinase assays were carried-out with cells after 2 and 6 days in culture. Immediately prior to the assay the cells in each well were rinsed 3x times with KRB containing 1mM cold Pi and ecto-protein kinase reactions in the same medium were initiated by adding [γ-^{32}P]ATP (0.1μM; 15μCi/well). After 10 min reaction in a final volume of 130μl, we removed 120μl of the medium, and the medium was subjected to low speed (1000xg) centrifugation for 5 min to yield S1 and P1. The first supenatant S1 was then spun at 150,000xg for 90 min to yield a soluble (S2) and particulate (P2) fractions. Aliquots from the remaining cells, pellets and concentrated supenatants were analysed by SDS-PAGE and autoradiography. In each experiment, 3-4 wells were processed individually. Six such experiments were carried-out to date. The audioradiographs revealed that 60-75% of the ^{32}P-phosphate incorporated into the 11.7K and 13K proteins were recovered in the high spin supernatant S2. Duplicate gels were stained by Coomassie blue and Biorad Silver Kit. Coomassie blue did not stain bands in the position of the 11.7K and 13K (seen in the AR), nor were they stained by silver in samples from the total reaction mixture. A faint band was stained by silver in the position of the 13K phosphorylated protein in samples of S1 and S2. Thus, upon phosphorylation by extracellular ATP, 60-75% of the 11.7 and 13K proteins dissociate from the cell surface and become soluble. Control experiments detected proteins (by staining) which dissociated to the soluble fraction after preincubation of cells for 10 min with cold ATP, but not in buffer without ATP. The solubilized fraction did not contain endogenous protein kinase activity, and the 11.7K and 13K were the <u>only</u> bands seen in the autoradiogram of S2 fraction obtained from cells reacted with [γ-^{32}P]ATP. Thus, during the phosphorylation reaction, these proteins selectively detach from the cell surface.

FORECAST

The finding that specific proteins detach from the surface of neurons upon phosphorylation by extracellular ATP may represent a novel signal operating during neurodevelopment and participating in processes underlying synaptic plasticity. The surface of the neighboring cells, matrix proteins and extracellular proenzymes (e.g. plasminogen) may be the targets of such signals. Availability of antibodies will enable direct examination of these potential physiological functions. In addition, this

new finding also provides a most promising procedure for purification of native proteins. According to Biorad consulting chemist, the lowest level of sensitivity for a faint band stained by their Silver Kit is 0.1 nanogram. Based on the amount of sample of S2 applied to the gel from a standard reaction we calculated that the yield of 13K in S2 is at least 0.28ng per 22×10^4 cells, or 37.7ng per telencephalon, or 130.8ng per brain (which is only 10X higher than the estimate obtained from calculations based on the specific activity of electroeluted band; a close agreement). Therefore, in this procedure we shall use dissociated cells from the brain of 4-5 dozen embryos per preparation to obtain the 11.7K and 13K surface phosphoproteins, in a soluble fraction. Several types of FPLC columns available for our Waters Advanced Protein Purification System would yield purified 11.7K and 13K proteins without the necessity for using denaturing detergents. The isolation of these proteins and preparation of antibodies against their native form are the main goals of our ongoing studies in this project. We believe that our progress to date proves the feasibility of achieving these goals.